

Attorney's Docket No.: GSF98-01

RELATED APPLICATIONS

10 BACKGROUND OF THE INVENTION

Mammary carcinoma is the most frequent tumor in women (Miller, A.D. and Bulbrook, R.D. *Int. J. Cancer* 37:173-177 (1986)). Up to now conventional therapy involves surgical removal of the primary tumor followed by a chemo- or radiation therapy. Depending on the tumor stage, the rate of relapse is quite high and has a fatal outcome in most cases.

• 10

It was thus quite unexpected when the inventors of the present invention found that the WAP regulatory sequence is

able to direct expression of a linked heterologous gene in primary human cells, including mammary carcinoma cells.

SUMMARY OF THE INVENTION

The invention then, inter alia, comprises the
5 following, alone or in combination:

A DNA construct comprising at least one therapeutic gene under transcriptional control of the WAP or MMTV regulatory sequences for the treatment of disorders or diseases of human mammary cells, including human mammary carcinoma;

a DNA construct as above wherein the regulatory sequence comprises the proximal 445 bp of the WAP promoter including the transcription initiation site.

a DNA construct as above wherein the regulatory
15 sequence contains the 320 bp XhoI/XbaI fragment of the WAP
promoter region;

a DNA construct as above wherein the regulatory sequence is the U3 region of MMTV;

a DNA construct as above wherein the regulatory
20 sequence contains the 0.6 Kb PstI MMTV promoter fragment;

a DNA construct as above which is a recombinant vector selected from viral and plasmid vectors;

a recombinant vector as above wherein said viral vector is selected from RNA and DNA viral vectors and said
25 plasmid vector is selected from eucaryotic expression
vectors;

5 a recombinant vector as above wherein the retroviral vector comprises a 5'LTR region of the structure U3-R-U5; at least one coding sequence for a therapeutic gene; and a 3'LTR region comprising a completely or partially deleted U3 region wherein said deleted region has been replaced by
10 a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region, said therapeutic gene being under transcriptional control of the WAP or MMTV regulatory sequences;

a construct as above, wherein said therapeutic gene is selected from the group consisting of genes which code for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450, cell cycle regulatory genes which code for proteins such as SDI, tumor supressor genes which code for proteins such as p53, antiproliferation genes which codes for proteins such as melittin, cecropin or cytokines such as IL-2;

a recombinant retroviral particle produced by culturing a packaging cell line harbouring a retroviral vector construct as above and one or more constructs coding

particle as above and a pharmaceutically acceptable carrier or diluent;

a pharmaceutical compositional for the treatment of disorders or diseases of human mammary cells, including
5 human mammary carcinoma comprising a cell line as above and a pharmaceutically acceptable carrier or diluent;

the use of the WAP or MMTV regulatory sequences for the expression of linked therapeutic genes in human mammary cells, including human mammary carcinoma cells;

10 the use as above wherein the regulatory sequence comprises the proximal 445 bp of the WAP promoter including the transcription initiation site;

the use as above wherein the regulatory sequence contain the 320 bp XhoI/XbaI fragment of the WAP promoter
15 region;

the use as above wherein the regulatory sequence is the U3 region of MMTV;

the use as above wherein the regulatory sequence contains the 0.6 Kb PstI MMTV promoter fragment;

20 the use as above wherein the therapeutic gene is selected from anti-tumor genes and cytokine genes;

the use as above, wherein said therapeutic gene is selected from the group consisting of genes which code for proteins such as Herpes Simplex Virus thymidine kinase,
25 cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450, cell cycle regulatory genes which code for proteins such as SDI, tumor supressor genes which code for proteins such as p53, antiproliferation genes

865060-9652060

a method for the treatment of human mammary carcinoma comprising administering to a human in need thereof cells as above; and

a method for the treatment of human mammary carcinoma
5 comprising implanting into a human in need thereof
encapsulated cells as above either in or nearby the site of
the tumor.

The therapeutic gene is preferably selected from one or more elements of the group consisting of antitumor genes and cytokine genes.

Said therapeutic genes are preferably selected from the group consisting of genes which code for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450, cell cycle regulatory genes such as SDI, or tumor suppressor genes which codes for proteins such as p53, or antiproliferation genes which code for proteins such as melittin, cecropin or cytokines such as IL-2.

Herpes Simplex Virus thymidine kinase, cytosine
20 deaminase, guanine phosphoribosyl transferase (gpt) and
cytochrome P 450 can be used in cancer treatment in
combination with a prodrug which is converted to its toxic
form by these enzymes.

The DNA constructs according to the invention can also carry a marker gene. Said marker genes are preferably selected from the group consisting of marker genes which code for proteins such as β -galactosidase, neomycin, alcohol dehydrogenase, luciferase, puromycin, hypoxanthine

phosphoribosyl transferase (HPRT), hygromycin, secreted alkaline phosphatase and green or blue fluorescent proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Figure 1: Primers A (SEQ ID NO: 1), B (SEQ ID NO: 2), and C (SEQ ID NO: 3) used for deletion of U3 of MLV and insertion of a SacII-Mlu polylinker in its place.
- Figure 2: The preparation of plasmid pSmaU3del.
- 10 Figure 3: The preparation of plasmids pBAGNU3del and pCON6.
- Figure 4: The preparation of plasmid pMMTV-BAG.
- Figure 5: Primers D (SEQ ID NO: 4) and E (SEQ ID NO: 5) used for amplification of the U3-region of MMTV.
- 15 Figure 6: Plasmid pMMTV-BAG.
- Figure 7: Primers F (SEQ ID NO: 6) and G (SEQ ID NO: 7) used for amplification of a sequence containing the proximal 445 bp of the WAP promoter and the first 143 bp's of human growth hormone.
- 20 Figure 8: The preparation of plasmid pWAP-BAG.
- Figure 9: Plasmid pWAP-BAG.
- Figure 10: β -Gal expression of vector constructs after infection of primary human mammary gland cells.
- 25

8650E0" 8650E060

DETAILED DESCRIPTION OF THE INVENTION

According to the invention it has surprisingly been found that the WAP and MMTV regulatory sequences are able to direct expression of a linked heterologous gene in
5 primary human mammary cells, including human mammary carcinoma cells.

It is well known that Whey Acidic Protein (WAP) gene expression is directed to the pregnant and lactating mammary gland of rodents (Günzburg, W.H., *Mol. Endocrin.*,
10 5:123-133 (1991); Hennighausen, L., *J. Cell. Biochem.* 49:325-332 (1992)). WAP gene expression is regulated both by hormone dependent and hormone independent mechanisms. A negative regulatory element (NRE) in the WAP promoter interacts with a NRE binding factor, NBF, which is present
15 in all cells unable to express WAP (Kolb, A.F. et al., *Biochem. Biophys. Res. Commun.*, 217:1045-1052 (1995)). It appears that the region of the WAP promoter which is required for mediating the mammary gland specificity is a 320 bp XhoI/XbaI restriction fragment (-413 to -93) (Kolb, A.F. et al., *Biochem. Biophys. Res. Commun.*, 217:1045-1052
20 (1995)). In addition certain experiments indicate that binding sites for NBF is present on a 0.6 Kb PstI MMTV promoter fragment (described in Salmons, B. et al., *Virology*, 144:101-114 (1985)) and may play a role in
25 regulating the mammary gland specificity of expression displayed by MMTV (Kolb, A.F., et al., *Biochem. Biophys. Res. Commun.*, 217:1045-1052 (1995)).

8550E0" 9655E060

The fact that the WAP and the MMTV are both derived from the rodent system may become an important safety feature because the use of human regulatory sequences in a retroviral vector may facilitate homologous recombinations
5 between the vector carried sequences and the corresponding cellular sequences and may cause genome instability.

The DNA constructs according to the invention carrying a therapeutic gene under transcriptional control of the WAP or MMTV regulatory sequences can be introduced into cells
10 using any known method for the introduction of genes into cells including by means of calcium phosphate precipitation (Graham et al., *Virology*, 52:456-467 (1973); Wigler et al., *Cell*, 777-785 (1979)), by means of electroporation (Neumann et al., *EMBO J.*, 1:841-845 (1982)), by microinjection
15 (Graessmann et al., *Meth. Enzym.*, 101:482-492 (1983)), by means of liposomes (Straubinger et al., *Methods in Enzym.*, 101:512-527 (1983)), by means of spheroblasts (Schaffner, *Proc. Natl. Acad. Sci. USA*, 77:2163-2167 (1980)) or by other methods known to those skilled in the art.

20 The DNA constructs according to the invention carrying a therapeutic gene under transcriptional control of the WAP or MMTV regulatory sequences are preferably introduced into cells using a recombinant viral vector. Such vectors are well known in the art and include retroviral vectors,
25 recombinant adenoviruses and recombinant adeno-associated viruses (Günzburg W.H., and Salmons, B., *Mol. Med. Today*, 1:410-417 (1995)) as well as herpes virus vectors.

8650E0-965E060

In a preferred embodiment of the invention the DNA construct according to the invention is a modified retroviral vector, which is used to deliver the therapeutic gene to cells, preferably in vivo. The great advantage of a retroviral system is that retroviruses can only infect dividing cells (especially rapid dividing cells such as tumor cells) and that the virus particles can be spread in the blood stream similarly to metastasising tumor cells, which will make it possible to eliminate micrometastases long before they can be detected by conventional methods.

Retroviral vector systems consist of two components:

1. the retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins have been replaced by therapeutic genes and/or marker genes to be transferred to the target cell. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2. a cell line that produces large quantities of the viral proteins however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and

[illegible][illegible][illegible][illegible]

5

15

25

5

10

20

25

Retroviral particles can be made by introducing a viral vector construct into a packaging cell which contain

the elements lacking in the vector construct (e.g. gag, pol, and env) which are necessary for the production of infectious retroviruses.

The packaging cell line can be selected from an
5 element of the group consisting of psi-2 (Mann, M. et al.,
Cell, 33:153-159 (1983)), psi-Crip (Danos, O. and Mulligan,
R.C., *Proc. Natl. Acad. Sci. USA*, 85:6460-6464 (1988)),
psi-AM (Cone R.D. and Mulligan, R.C., *Proc. Natl. Acad.*
Sci. USA, 81:6349-6353 (1984)), GP+E-86 (Markowitz, D. et
10 al., *J. Virol.*, 62:1120-1124 (1988a), PA317 (Miller A.D.
and Buttimore, C., *Mol. Cell. Biol.*, 6:2895-2902 (1986)),
GP+envAM-12 (Markowitz, D. et al., *Virology*, 167:400-406
(1988b), Bosc 23, Bing (Pear, W.S. et al., *Proc. Natl.*
Acad. Sci. USA, 90:8392-8396 (1993)) or FLYA13, FLYRD 18
15 (Casset F.L. et al., *J. Virol.*, 69:7430-7436 (1995)) or of
any of these transfected with recombinant constructs
allowing expression of surface proteins from other
enveloped viruses. Such pseudotyped retroviral particles
are described in PCT/EP96/01348.

20 In particular preferred embodiment, the packaging cell
line is made from human cells, e.g. HT10808 cells (WO-A1-
9621014), 293 (Graham et al., *J. Gen. Virol.*, 36:59 (1977))
or mink cell lines thereby allowing production of
recombinant retroviruses that are capable of surviving
25 inactivation by human serum.

According to the invention the term "polylinker" is
used for a short stretch of artificially synthesized DNA

865060" 9655060

which carries unique restriction sites allowing the easy insertion of any promoter or DNA segment.

The term "heterologous" is used for any combination of DNA sequences that is not normally found intimately associated in nature.

PRESERVATION AND ADMINISTRATION OF VIRAL PARTICLES

Recombinant retrovirus which has been purified or concentrated may be preserved by first adding a sufficient amount of formulation buffer to the media containing the recombina
10 nt retrovirus, in order to form an aqueous suspension. The formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino
15 acids.

The recombinant retrovirus can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude recombinant retrovirus described above may be clarified by passing it through a filter, and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Northborough, MA). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated to remove excess media components and establish the recombinant retrovirus in a more desirable buffered solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified recombinant

retrovirus is eluted. A sufficient amount of formulation buffer is added to this eluate to reach a desired final concentration of the constituents and to minimally dilute the recombinant retrovirus, and the aqueous suspension is then stored, preferably at -70°C or immediately dried. As noted above, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The crude recombinant retrovirus can also be purified by ion exchange column chromatography. In general, the crude recombinant retrovirus is clarified by passing it through a filter, and the filtrate loaded onto a column containing a highly sulfonated cellulose matrix. The recombinant retrovirus is eluted from the column in purified form by using a high salt buffer. The high salt buffer is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant retrovirus and the aqueous suspension is either dried immediately or stored, preferably at -70°C .

The aqueous suspension in crude or purified form can be dried by lyophilisation or evaporation at ambient temperature. Specifically, lyophilisation involves the steps of cooling the aqueous suspension below the glass transition temperature or below the eutectic point

temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilised retrovirus. Once lyophilised, the recombinant retrovirus is stable and may be stored at -20°C to 25°C, as discussed in more detail below.

Within the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Water can also be removed through spray drying.

The aqueous solutions used for formulation, as previously described, are composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the recombinant retrovirus upon freezing and lyophilization, or drying through evaporation.

The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilised or dried state.

Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin.

The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of

865000-000000

the cooled aqueous suspension and while in the lyophilised state.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers
5 may be used, depending on the pH range desired, preferably between 7.0 and 7.8.

Aqueous solutions for the formulation of recombinant retroviruses are described in detail in WO-A2-96121014.

In addition, it is preferable that the aqueous
10 solution contain a neutral salt which is used to adjust the final formulated recombinant retrovirus to an appropriate iso-osmotic salt concentration.

Lyophilized or dehydrated retroviruses may be reconstituted using a variety of substances, but are
15 preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the
20 reconstituted retrovirus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted retrovirus. Lyophilized or dehydrated recombinant retrovirus may be reconstituted with
25 any convenient volume of water or the reconstituting agents that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

865020-365060

Recombinant retroviral particles may be administered to a wide variety of locations including, for example, into sites such as an organ or to a site of a tumor. Within other embodiments, the recombinant retrovirus may be
5 administered orally, intravenously, buccal/sublingual, intraperitoneally, or subcutaneously. The daily dosage depends upon the exact mode of administration, form in which administered, the indication toward which the administration is directed, the subject involved and the
10 body weight of the subject involved, and further the preference and experience of the physician in charge.

The routes of administration described herein may be accomplished simply by direct administration using a needle, catheter or related device. In particular, within
15 certain embodiments of the invention, one or more dosages may be administered directly.

In another embodiment of the invention a cell line according to the invention producing either a therapeutic polypeptide or viral particles carrying a therapeutic gene
20 under the transcriptional control of the WAP or MMTV regulatory sequences is encapsulated in a porous membrane which is permeable to the therapeutic polypeptide or viral particles produced (Stange, J. et al., *Biomat. Art. Cells & Immob., Biotech.*, 21(3):343-352 (1993); Dautzenberg, H., et
25 al., *Biomat. Art. Cells & Immob., Biotech.*, 21:(3):399-405 (1993); Merten O.W. et al., *Cytotechnology*, 121-130 (1991) and UK Patent Application 2 135 954)).

8650E0 9655E060

The encapsulated cells according to the invention can be prepared for example by suspending the cells in an aqueous solution of a polyelectrolyte (e.g. selected from sulphate group-containing polysaccharides or polysaccharide derivatives or of sulphonate group containing synthetic polymers), whereafter the suspension in the form of preformed particles is introduced into a precipitation bath containing an aqueous solution of a counter-charged polyelectrolyte (such as for example a polymer with quaternary ammonium groups).

Sulphate group-containing polysaccharides or polysaccharide derivatives include cellulose sulphate, cellulose acetate sulphate, carboxymethylcellulose sulphate, dextran sulphate or starch sulphate in the form of a salt, especially a sodium salt. The sulphonate group-containing synthetic polymer can be a polystyrene sulphonate salt, preferably a sodium salt.

Polymers with quaternary ammonium groups includes polydimethyldiallylammonium or polyvinylbenzyltrimethylammonium, in the form of a salt thereof, preferably a chloride salt. Such capsules are preferably prepared by suspending the cells of the invention in a solution containing 0.5-50%, preferably 2-5% sodium cellulose sulphate and 5% fetal calf serum in PBS. This suspension is then dropped by a dispensing system (e.g. air-jet system or piezoelectric system) while stirring into a precipitation bath containing 0.5%-50%, preferably 2-10%, or most preferred 3% polydimethyl-diallylammonium chloride

in PBS. Capsule formation occurs within milliseconds and the capsules containing cells are kept in the precipitation bath for 30 seconds to 5 minutes and then washed. The rapidity of this method ensures that the cells are not unduly stressed during the whole procedure (Stange, J. et al., *Biomat. Art. Cells & Immob., Biotech* 21(3):343-352 (1993)).

The encapsulated cells can be cultivated in a normal cell culture medium (the nature of which depends on the encapsulated cells) at standard conditions of humidity, temperature and CO₂ concentration. During this culture period production of therapeutic polypeptides and viral particles from the capsules into the cell culture medium can be demonstrated. Production of viral particles can be demonstrated using for RT-PCR technology or by transfer of cell free (0.45 μ m filtered) supernatant to target cells followed by the demonstration of viral infection by assay for the activity of marker proteins encoded by genes carried by the viral vector construct contained within the viral particle. If the marker gene carried by the viral vector is a gene conferring resistance to a specific compound upon the target cell or the product of which is easily assayed on a cell to cell basis e.g. green or blue fluorescent protein, the titre of virus produced by the system can be ascertained.

After a suitable period in culture (normally not less than 1 hour and not exceeding 30 days), the cell containing capsules can be surgically implanted either directly, or by

8650E0"9655E060

injection using a syringe into various areas of the body including the breast.

The following examples will illustrate the invention further. The example is however in no way intended to
5 limit the scope of the present invention as obvious modifications will be apparent, and still other modifications and substitutions will be apparent to anyone skilled in the art.

The recombinant DNA methods employed in practicing the
10 present invention are standard procedures, well known to those skilled in the art, and described in detail, for example, in "Molecular Cloning" (Sambrook, J., et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York, USA (1989)) and in "A Practical Guide to Molecular
15 Cloning" (Perbal, B., *A Practical Guide to Molecular Cloning*, John Wiley & Sons (1994)).

EXAMPLE 1

DELETION OF THE U3 REGION AND INSERTION OF A POLYLINKER

In the murine leukemia virus (MLV) retroviral vector
20 known as BAG (Price, J., et al., *Proc. Natl. Acad. Sci. USA*, 84:156-160 (1987)) the β -galactosidase gene is driven by the promiscuous (i.e. non-tissue specific) MLV promoter in the U3 region of the LTR. According to the present invention a derivative of the BAG vector has been
25 constructed in which the MLV promoter (U3) located within the 3'LTR except the inverted repeat has been deleted by PCR and replaced by a polylinker. The BAG vector lacking

8650E0-965E060

the U3 is expressed from the MLV promoter (U3) within the 5'LTR when introduced into a packaging cell line. As a result of the rearrangements occurring in the retroviral genome during its life cycle, following infection of its target cell, the polylinker will be duplicated at both ends of the retroviral genome as described in WO-A1-9607748. Thereby a retroviral vector can be constructed in which the expression of the β -galactosidase gene of BAG will be controlled by any heterologous promoter inserted into the polylinker.

As a template for PCR we used pBAGN a plasmid carrying a derivative of the BAG construct carrying only one LTR, created by an NheI digest of the original pBAG followed by a self-ligation of the 7018bp fragment.

The 3' end of primer A is complementary to the R-region of the LTR (Figure 1). The 5'-extension contains an artificial (art.) polylinker and an artificial inverted repeat (IR(art.)). Primer B is complementary to the U5 region of the LTR (Figure 1). After 35 cycles of annealing at 47°C and extension at 60°C, a 140bp product was obtained, which was used as a template for the second PCR. In this reaction a Clal site and an artificial (+)PBS was added 5' of the IR-region using primer C (Figure 1) in combination with primer B. Annealing was carried out at 53°C and extension at 72°C. After 35 cycles a 163bp product was obtained, which was digested with Clal and SmaI and ligated to a 2722bp Clal/SmaI fragment of pSmaI (Figure 2). The resulting plasmid pSmaIU3del (2792bp) was

5 pSma1U3del as template.

10 full U3-minus retroviral vector was used as a basis for

polylinker region or the modified BAG vector.

15 EXAMPLE 2

Cloning of pMMTVgal

20 containing an element that directs expression to the

as template with primers D and E.

25 The 3' end of primer D is complementary to the 5' end

extension (Figure 5). The 3' end of primer E is

complementary to the 3' end of the MMTV U3 region and has a MluI site in its 5' extension (Figure 5).

After 35 cycles of annealing at 49°C and extension at 72°C, a 1229bp product was obtained, digested with SacII and MluI and ligated to the SacII/MluI digested vector pCON6. The resulting plasmid p125.6 (5305bp) (Figure 4) was digested with XbaI and HindIII and the 4187 bp fragment ligated to the 4190bp fragment of pBAGN containing the β -galactosidase gene to give the plasmid pMMTV-BAG (8377bp) (Figure 4) in which the β -galactosidase gene is under the transcriptional control of the MLV promoter after transfection, and under the MMTV promoter after infection (Figure 6).

EXAMPLE 3

Cloning of the Whey Acidic Protein (WAP) promoter region encompassing the proximal 445 bp of the WAP promoter including the transcription initiation site.

A plasmid, pWAPBAG containing the β -galactosidase gene under transcriptional control of the proximal 445 bp's of the WAP promoter was prepared by amplification of a sequence comprising the proximal 445 bp's of the WAP promoter and the first 143 bp's of the human growth hormone (HGH). The sequences were amplified from pWAP2-HGH (Günzburg W.H. et al., *Molecular Endocrinology*, 5:123-133 (1991)) by PCR using primers F and G. (Figure 7). Both primers carried SacII and MluI recognition sites as terminal sequences. The amplified 606 bp product and pCON6

were digested with SacI and MluI and the 4094 bp fragment of the vector as well as the PCR product was ligated together to create pWAP.6. The β -galactosidase (β -gal) gene of E.coli was cloned into the resulting vector pWAP.6 (4687 bp) (Figure 8); pWAP.6 as well as pBAGN were digested with BamHI and the linearized vector fragment as well as the 3072 bp β -gal fragment of pBAGN were ligated together. The resulting plasmid was pWAPBAG (Figure 9), which is a ProCon vector in which the 3' 445 bp's containing the WAP-NRE, as well as the 5' 143 bp's of the HGH coding sequence, were inserted in place of the U3 region in the 3' LTR.

EXAMPLE 4

The control of the B-galactosidase gene expression under the transcriptional control of the WAP and MMTV promoters inserted into the polylinker has been validated by infection studies using the constructed MMTV and WAP retroviral vectors to infect various cells.

To produce retroviral vector particles, the MMTV and WAP ProCon vectors have been transfected into the packaging cell line PA317 (Miller, A.B. and Buttimore, C., *Mol. Cell. Biol.*, 6:2895-2902 (1986)). After selection for neomycin resistance, which is encoded by the vector, stable populations and clones of recombinant ProCon virus producing cells were obtained. Virus containing supernatant from these populations was used to infect explanted normal primary human mammary tissue obtained from reduction mammoplasties. Since it is known that the WAP

8550E0" 9655E060

and MMTV promoters are responsive to pregnancy hormones, the tissue was cultivated in the presence of 10^{-6} M dexamethasone, insulin ($1\mu\text{g/ml}$), EGF (5ng/ml) and Cortisol (0.1 g/ml). The expression of the marker gene was

5 determined by a quantitative β -gal assay which is based on the detection of β -galactosidase activity by chemiluminescence.

β -gal assay:

The levels of β -galactosidase activity were assayed

10 using the Tropix kit according to the manufacturers' instructions. The cells were trypsinised, washed twice with PBS and lysed. The protein concentration was determined by a modified Lowry (BioRad DC protein assay). 5 and $10\text{ }\mu\text{g}$ of protein was used for the β -gal assay. The

15 kit uses a substrate that is cleaved by the enzyme and thus the amount of enzyme activity is proportional to the amount of light produced. Expression was quantified by measuring chemiluminescence in a Berthold AutoLumat 953 (EG+G Berthold).

20 Detection of infected cells by histochemical staining was performed as outlined previously ([Cepko, 1989]). Briefly, the cells were washed with chilled PBS and then fixed with a 2% paraformaldehyde solution for 20 mins. After extensive washing with PBS, the cells were incubated

25 in a solution containing the substrate X-gal ($20\text{ mM K}_3\text{FeCN}_6$, $20\text{mM K}_4\text{FeCN}_6 \cdot 3\text{H}_2\text{O}$, 2mM MgCl_2 and 1 mg/ml X-gal) for at least 2 hours at 37°C .

8650E0 965E060

In all the experiments the original, non-tissue specific BAG-vector was used as a positive control. After infection of normal primary human mammary gland cells with BAG, WAPBAG and MMTVBAG, samples showed β -galactosidase expression (Figure 10) in three independent experiments. It has thus been demonstrated for the first time that the WAP regulatory elements as well as the MMTV-U3 region can drive the expression of a gene within a MLV retroviral vector in primary human mammary gland cells.

10 EXAMPLE 5

Cytochrome P450 catalyses the hydroxylation of the commonly used cancer prodrugs cyclophosphamide (CPA) and ifosfamide to their active toxic forms. Normally the expression of the patient's endogenous cytochrome P450 gene is limited to the liver, and anti-tumor effects of systemically applied CPA's depend upon the subsequent systemic distribution of toxic drug metabolites from the liver. This has led to toxicity problems since the activated drug not only affects the tumor but also affects other normal patient tissues such as bone marrow and kidney.

A therapeutic approach, where the cytochrome P450 gene is selectively introduced directly into tumor cells, and overexpressed in these cells, would circumvent this problem. Toxic metabolites produced from the transduced tumor cells affect surrounding non-transduced tumor cells in a concentration gradient dependent manner. An

additional advantage of the cytochrome P-450/CPA system is the lack of dependency upon cell replication for cytotoxic effects on the surrounding cells. This is because one of the active metabolites generated causes interstrand
5 crosslinks regardless of the cell cycle phase. Later on, during DNA synthesis, these interstrand crosslinks result in cell death.

Construction of a retroviral vector carrying the rat cytochrome P450 gene under control of the WAP regulatory
10 sequence:

To yield the rat cytochrome P450 2B1 gene, cells of the rat hepatoma cell line HTC were lysed with solution D (4M guanidium thiocyanate, 25mM sodium citrate pH7, 0.5%N-laurylsarcosine sodium, 0.1M 2-mercaptoethanol) and total
15 RNA extracted by adding 1/15 volume of 3M sodium acetate, in the same volume of watersaturated phenol and 1/5 volume of chloroform/isoamylalcohol (49:1) were added and the whole mixture mixed vigorously. After 15 minutes on ice the extract was centrifuged 20 minutes at 4°C and 10,000g.
20 The RNA in the Aqueous phase was precipitated with one volume of isopropanol for 30 minutes at 20°C and centrifuged at 10,000g at 4°C. The pellet was washed in 70% ethanol and left at room temperature for 15 minutes. After 5 minute centrifugation at 4°C and 10,000g the pellet
25 was dried in a vacuum dryer and redissolved in 0.5% SDS solution.

The extracted RNA is reverse transcribed using the protocol for cDNA synthesis (Pharmacia). The resulting

8550E0 355E060

cDNA is used as template for PCR. The primers are designed so that they contained a BamHI restriction site (underlined) in the lefthand primer (e.g. 5'AAGCCGGATCCCTGGAGAGCATGCAC-3' (SEQ ID NO: 8)) and a BamHI site (underlined) in the righthand primer (e.g. 5'CGATTAGGATCCCTGCCTCA-3' (SEQ ID NO: 9)). Both primers have additional bases at the 5'-end for higher efficiency of cleavage by the relevant restriction enzyme. The 1562 bp-product is digested with BamHI and the fragment obtained is containing the gene for P450 is ligated into the BamHI digested plasmid pWAP.6 (example 3).

One day before lipofection 3×10^5 retroviral packaging cells are seeded into 6cm petri or culture dishes. On the day of infection $2 \mu\text{g}$ of the vector encoding cytochrome P450 under transcriptional control of the WAP regulatory sequence are mixed with $100 \mu\text{l}$ serum free media. In parallel $15 \mu\text{l}$ of Lipofectamine (Gibco BRL) is mixed with $100 \mu\text{l}$ serumfree media. The plasmid containing solution is added to the Lipofectamine-mix and incubated for 45 minutes. After 35 minutes the cells are washed once with 2ml serum free media. $800 \mu\text{l}$ of serum free media were added to the lipofection-mix and the resulting 1ml is put onto the prepared cells. After 6 hours 1ml Dulbecco's modified Eagles medium containing 10% FCS is added. The next day the cells are trypsinized and 1:10 diluted and seeded on a 100mm dish. After 24h the media is replaced with medium containing the neomycin analog G418. Single cell clones or

cell populations are isolated and analysed for expression of cytochrome P450.

ENCAPSULATION OF CELLS AND IMPLANTATION OF ENCAPSULATED CELLS

5 The retroviral vector producing packaging cells are suspending in 1ml of 0.5-50%, but preferably 2-5%, anionic polymer (e.g. sodium cellulose sulphate) solution which also contains 5% fetal calf serum. This suspension is then dropped by a dispensing system (e.g. A-jet system or
10 piezoelectric system) into a precipitation bath containing a stirred 0.5%-50% polymeric polycation (e.g. polydimethyldiallylammonium). The capsule formation occurs within milliseconds and the capsules containing cells are kept in the precipitation bath for 30 seconds to 5 minutes
15 and then washed. The rapidity of this method ensures that the cells are not unduly stressed during the whole procedure. (Stange, L. et al., *Biomat. Art. Cells & Immob. Biotech.*, 21(3):343-352 (1993)).

 The capsules producing viral particles are implanted
20 in or around the mammary tumor in capsules thereby ensuring continuous release of virus.

 Alternatively, the virus could be introduced by multiple direct injections into the mammary tumor. Systemic or local administration of cyclophosphamide or
25 ifosfamide will result in local conversion of these compounds to their toxic forms leading to ablation of tumor cells.